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16. ABSTRACT

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EMERGING METHODOLOGIES FOR ASSESSMENT OF COMPLEX MIXTURES: APPLICATION OF BIOASSAYS IN THE INTEGRATED AIR CANCER PROJECT

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The assessment of complex mixtures of environmental pollutants requires new interdisciplinary strategies. Integration of bioassay methodologies into these strategies is an important tool that provides direct evidence of the toxicity of a mixture. Short-term genetic bioassays are now widely used in the cancer assessment of complex mixtures. New and emerging interdisciplinary methodologies for assessing complex mixtures using bioassays are illustrated by the U.S. EPA's Integrated Air Cancer Project (IACP). The goals of this project are to identify the major airborne carcinogens and their emission sources and to improve the methodology and data available for human exposure and risk assessment from airborne carcinogens. The research effort is focused primarily on characterizing the impact of complex mixtures of products of incomplete combustion, including gaseous, semi-volatile, and particle-bound organic species. Short-term genetic bioassays have been integrated into several analysis strategies, including environmental biomonitoring; bioassay-directed fractionation/characterization; transformation studies; source apportionment; and exposure, dosimetry, and risk assessment.

INTRODUCTION

Recognition that most human exposure to environmental chemicals occurs as exposures to complex mixtures has stimulated research on the toxicology of such mixtures (NRC, 1988). Air pollution, particularly involving mixtures emitted from combustion sources, is a major contributor to human exposure to complex mixtures (Holmberg

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2. Key words: air pollution, bioassay-directed fractionation, cancer, combustion emissions, complex mixtures, mutagenesis, source apportionment.

3. Abbreviations: IACP = Integrated Air Cancer Project.

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and Ahlborg, 1983; Lewtas, 1988). Combustion emissions consist of a complex mixture of gases and particles (soot) containing both inorganic and organic components. The particle phase of air pollution from combustion emissions contains an organic extractable tar that is similar to the coal tars from soots demonstrated to induce scrotal cancer in chimney sweeps and tumors in animals in the early 1900s (IARC, 1985). The extractable organics from urban air particles and many combustion sources are genotoxic (i.e., induce mutations and other genetic damage) in short-term bioassays and induce tumors in rodents (Lewtas, 1985, 1988).

Many scientific questions regarding the relationship between air pollution and human cancer remain unanswered. This is true primarily because estimates of human cancer caused by air pollution are highly refractory to the traditional techniques of epidemiology and exposure and risk assessment. The length of time (decades) between exposure to airborne carcinogens and diagnosis of cancer precludes both meaningful prospective epidemiological studies of human populations and quantitative estimates of the risk from exposure to mixtures of airborne carcinogens. Retrospective studies are complicated by lack of knowledge as to when the observed cancer was initiated. Because most nonoccupational cancers are not specific and could have multiple causes associating an observed cancer with a given putative agent has not been successful. In 1977 and again in 1982, international expert groups concluded that air pollution arising from combustion products of fossil fuels, probably acting together with cigarette smoke, has been responsible for 10% of all cancers in the United States (Holmberg and Ahlborg, 1983; Report of the Task Group, 1978). This estimate is a "best guess" based on studies of urban versus rural cancer rates. Doll and Peto (1981), in a review of cancer causes in the United States, suggest that a much lower rate of 2% of cancer deaths could be attributed to pollution. Karch and Schneiderman (1981), on the other extreme, suggest that past analyses have overestimated the contribution of smoking and underestimated the multicausal nature of cancer. They estimate that at least 11% and more likely 21% of lung cancer is related to air pollution. Regardless of which of these estimates proves to be the most accurate, there is certainly reason to believe that the carcinogenic potential of air pollution may be a serious public health problem.

The U.S. Environmental Protection Agency (EPA) has undertaken a major interdisciplinary research program, the Integrated Air Cancer Project (IACP), directed specifically at clarifying the exposure, risk, and sources of carcinogens in the air, using both biological and chemical methods. As a part of the IACP, new methodologies are being developed to estimate the potential cancer risk associated with human exposure to air pollution resulting from direct and transformed combustion emissions. The EPA has the responsibility and authority to regulate the emission of carcinogens into the air (e.g., Section 112, Clean Air Act); however, identifying which carcinogens and emission sources are of greatest potential human risk is problematic and remains an important research goal. The IACP focuses on identifying those species present in the air that are mutagenic and likely to be carcinogenic and attempts to determine how

they came to be present in the atmosphere. To accomplish this, the IACP has brought together the multidisciplinary talents of chemists, physicists, engineers, and toxicologists to conduct coordinated field and laboratory studies on combustion emission sources and their atmospheric transport and transformation, human exposures, and the mutagenic and carcinogenic activity resulting from these exposures.

INTEGRATED AIR CANCER PROJECT GOALS AND STRATEGY

Identify the Principal Airborne Carcinogens. Airborne carcinogens include gaseous or volatile organic compounds, semi-volatile organic compounds, and particulate-bound organic compounds (e.g., polycyclic organic matter). The identities and relative contributions to the total atmospheric burden of carcinogens by many of the organic chemicals present in the atmosphere are not yet known. The IACP is developing the methods and data necessary to characterize these species through the combined use of chemical analysis and genetic bioassay methods. The specific chemicals or chemical classes that are mutagenic and carcinogenic compounds are being identified, and their concentrations in the environment measured. These carcinogens may be direct emissions from their sources or the transformation products produced from reactions of simple hydrocarbons or complex aromatic compounds with acidic and oxidizing gases in the atmosphere. The complexity of the problem requires that an integrated approach be used. To determine the source of specific airborne carcinogens, source emissions need to be characterized, as well as the transformation products from those emissions.

Determine Which Emission Sources Are Major Contributors of Carcinogens to Ambient Air. To decrease human exposure to carcinogens, the sources of the carcinogenic compounds or their precursors have to be identified. Field studies with simultaneous emission characterizations and ambient monitoring, followed by source apportionment calculations, are being conducted to determine which emission sources are the major contributors of carcinogens to the ambient air. Source emissions and ambient samples are being characterized using both chemical and biological (mutagenicity and carcinogenicity) methods.

Improve the Estimate of Human Exposure and Comparative Human Cancer Risk from Specific Air Pollution Emission Sources. A comparative bioassay potency methodology is being developed to evaluate and utilize short-term mutagenesis and animal carcinogenesis data on emission sources to improve the estimate of relative human cancer risk from specific air pollution emission sources (Lewtas, 1985, 1988). In addition, better total human exposure estimates will be developed for these complex emission products and individual carcinogens, including transformation products. Characterization of indoor microenvironments is an important component of efforts to improve human exposure assessment of combustion emissions.

Strategy. The long-range goals of the IACP are being approached through a stepwise series of field studies progressing from relatively simple to more complex airsheds. Current data indicate that motor vehicles and residential home heating sources are significant contributors to the mutagenic activity of ambient air samples. Therefore, the initial phases of IACP were aimed at quantifying carcinogens emitted from residential woodstoves and motor vehicles. Communities were selected with relatively simple airsheds in which a significant percentage of the homes use wood as the major heating fuel. The initial IACP studies conducted in Raleigh, NC, and Albuquerque, NM, emphasized field and laboratory evaluation to select sampling and analysis methodologies for the first major field study conducted in Boise, ID, in the winter of 1986–87 (Highsmith, in press). Transformation studies in laboratory simulations are assessing the role of atmospheric chemistry in altering the chemical composition and mutagenicity of emitted chemicals. Simulations of field study conditions will be used to determine the critical factors and chemical processes that transform the species between the source and the receptor.

TECHNICAL APPROACH

Integrated Field Studies. The field studies were designed to simultaneously sample and characterize the emissions at the source (e.g., woodstoves or motor vehicles) in ambient air near specific sources and in the ambient air distant from an aggregate of sources (Highsmith et al., 1987). Sampling indoors and outdoors of homes both with and without woodstoves was designed to provide an indication of total human exposure. In general, source and ambient emission measurements were made during heating and nonheating periods as well as separate daytime and nighttime sampling periods to differentiate between wood combustion emissions and those from motor vehicles. Indoor, ambient, and source emissions were collected simultaneously and analyzed using the same methods to provide a valid comparison between fresh emissions and chemicals at the receptor. Neighborhood survey data of motor vehicle and residential heating use was also recorded. Meteorological measurements and tracer studies were conducted throughout field tests as input to the source apportionment and atmospheric transformation studies.

The initial IACP field study in Raleigh, NC, included major components of methods development and evaluation. Factors such as face velocity, sample integrity, and sample extraction and preparation methods were evaluated, and standard procedures were developed for subsequent studies. Sampling methods were developed so that comparable samples could be taken simultaneously from woodstove sources and ambient air (indoors and outdoors). To collect large (gram) quantities of ambient air particulate matter for bioassay-directed fractionation/characterization and carcinogenesis studies, new high-volume samplers have been designed (Burton et al., 1987). The sampling protocols take into consideration techniques necessary to collect particulate material as well as volatile, semi-volatile, and condensable organic material.

New sampling and analysis methods were developed to prepare, sample, and extract large quantities of XAD-2 resin for organic characterization and bioassay studies. The results of the study included sampling protocols for source, ambient, and microenvironment collection methods.

APPLICATION OF BIOASSAYS IN THE ANALYSIS STRATEGIES

Biomonitoring. The development of short-term genetic bioassays in the mid-1970s rapidly led to their use in environmental monitoring. The essential elements of these bioassays that facilitate their application to environmental monitoring are their simplicity, rapidity, low cost, and small sample size requirements. The genetic bioassay most widely used has been the *Salmonella typhimurium* plate incorporation assay that Dr. Bruce Ames described in 1975 and initially validated as a bioassay to screen for carcinogens (McCann et al., 1975). This bioassay was rapidly adopted around the world and applied to environmental studies of complex mixtures. In air pollution monitoring studies, this bioassay was used to detect and quantitate the mutagenic activity associated with airborne particulate matter.

The relatively small sample mass requirements (milligram quantities) was one of the most important attributes that facilitated application of bacterial mutation assays to environmental monitoring. Recent advances in exposure monitoring have resulted in the need for assay methods that can be applied to even smaller (microgram) quantities of environmental samples. Microsuspension bacterial mutation assays have been developed to meet these needs through simple modifications of the *S. typhimurium* preincubation assay in which the assay is conducted in a ten- to 100-fold smaller volume (Kado et al., 1983; Lewtas, 1987). Microsuspension assays have been applied to indoor air pollution monitoring, human exposure monitoring, and in monitoring trends in air pollution over relatively short time periods (Lewtas, in press).

The IACP is extensively using short-term genetic bioassays in biomonitoring the mutagenic activity of airborne organics as particle extracts, semi-volatile organics (collected on XAD-2 resin), or gases (by direct exposure). Both the well-standardized Ames *S. typhimurium* plate incorporation assay and microsuspension assays are being used in the Project (Claxton et al., 1987). Bioassays are identifying the major carcinogenic components (e.g., gases, semi-volatile organics, or particles), chemical classes, and specific compounds in the airsheds being studied. For the first time in source apportionment studies, bioassays are being used extensively to determine which emission sources are the major contributors of carcinogens to ambient air. Finally, micromutagenesis bioassays are being conducted to determine human exposure to mutagens both indoors and outdoors.

Bioassay Directed Fractionation/Characterization. This approach has been used successfully to identify potential carcinogens in complex mixtures, including urban air

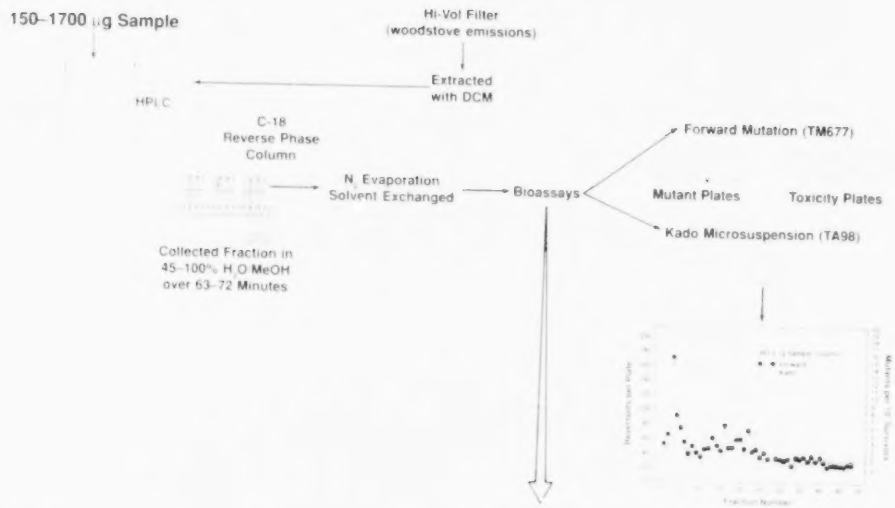

FIGURE 1. Illustration of new bioassay-directed fraction methodologies combining high pressure liquid chromatographic (HPLC) fractionation with bioassay analysis using two microsuspension mutagenesis assays. Both bioassays are conducted in *Salmonella typhimurium* strains. The forward mutation assay is conducted in TM677 and measures both mutants and survivors (Lewtas, 1987). The Kado microsuspension assay was performed in TA98 as described by Kado (1983) and Lewtas (1987). The mutagram bioassay chromatogram shown is for a wood stove source sample from the IACP, as reported in Lewtas (1988).

particles, and has been reviewed by Schuetzle and Lewtas (1986). The complex mixtures are fractionated and each fraction is bioassayed. Mutagenically active fractions are further fractionated, bioassayed, and characterized until the major class or specific compounds responsible for the mutagenicity are identified. The IACP is using the standard Ames *S. typhimurium* plate incorporation mutagenicity assay and the new micromutagenesis bioassays, coupled directly to analytical fractionation procedures (see Fig. 1), to identify the airborne mutagens in the IACP airsheds.

Gaseous Mutagens Formed By Atmospheric Transformation. An increasing number of laboratory and field studies have suggested that there are unidentified reactive compounds present in ambient air that cause increased mutagenic responses in both the vapor and aerosol phases. Chemical analysis has not fully identified many of these compounds, but the chemical class information suggests that the target compounds may be the partially-oxygenated or partially-nitrogenated reaction and transformation products of emitted organics. Recent studies have demonstrated that exposure of many organic hydrocarbons to photochemical reaction conditions increases the mutagenicity of the emissions, especially the gaseous components (Claxton, 1985).

The transformation studies carried out as part of the IACP are using a large reaction and atmospheric simulation chamber (mobile aerosol reaction chamber) to produce

TABLE 1
Effect of Atmospheric Transformation on the Mutagenicity of Wood Smoke[a]

	Mutagenic Potency (Revertants/μg)		Concentration of Mutagens (Revertants/m^3)	
	Reactants	Transformation Products	Reactants	Transformation Products
Gas phase	0	0.2-1.5	0	2,130-16,000
Particle phase	0.2	0.9	80	730

[a]Mutagenic activity measured in *Salmoella typhimurium* TA98 as described by Claxton (1985) with 22.7-m^3 Telfon smog reaction chamber and bioassay exposure chambers in atmospheric transformation studies described by Kleindienst et al., 1986.

irradiation products of air mixtures like those found in typical urban environments and in the IACP field study. The simulation chamber, a continuously stirred tank reactor, produces consistent conditions that permit a reproducible exposure of the bioassay test system. This system also provides large quantities of the transformation products for sampling and chemical analysis. Bioassay studies have been conducted on unreacted combustion source emissions and on the irradiation and transformation products (Cupitt et al., 1987). Studies of woodstove emissions show that atmospheric transformation substantially increases the mutagenic activity of the gaseous components (Table 1).

Source Apportionment of Airborne Mutagens. Source apportionment is a combination of mathematical and analytical procedures that determine the contribution of specific emission sources to air pollution. Several methods have been used to apportion the contributions of source emissions to ambient air quality, including emission inventory methods, source dispersion models, and receptor models (Gordon, 1988). The receptor-modeling approach to source apportionment that is being used in the IACP is especially effective where the number of sources are small and well characterized. In addition, emissions from motor vehicles and wood combustion contain unique elemental tracers that improve the accuracy of the source apportionment calculations.

Source apportionment of mutagenic activity and the use of organics as tracer species in receptor-model studies have not been evaluated extensively and are a major component of the IACP (Lewis et al., 1988). The unique feature in the receptor-modeling studies in the IACP is that mutagenicity data from biomonitoring is being used directly in the model, together with elemental tracers (e.g., potassium and lead). This has made possible the direct apportionment of mutagenic activity in the wood smoke impacted airsheds as shown in Table 2. Radiocarbon dating has been used to validate this receptor model (Klouda et al., 1987). Organic tracers (Merrill, 1987) are being investigated for use in the source apportionment calculations.

TABLE 2
Average Ambient Concentrations and TA98 +S9 Mutagenic Potencies Attributed to Wood Smoke and Motor Vehicles[a]

	Wood Smoke	Motor Vehicles	Other Sources	Total
Albuquerque Results				
EMO[b], $\mu g/m^3$	15 ± 1	3 ± .9	1 ± 0.9	19
Mutagenicity, rev/m^3	19 ± 2	11 ± 3	3 ± 3.1	32
Potency, rev/μg EOM	1.3 ± 0.3	3.7 ± 1.5		
Raleigh Results[c]				
EMO[b], $\mu g/m^3$	16 ± 0.5	1 ± 0.3	0.1 ± 1	17
Mutagenicity, rev/m^3	12 ± 2.9	4 ± 1	1 ± 1	18
Potency, rev/μg EOM	.78 ± 0.07	3.7 ± 1.5		

[a]Determined from receptor modeling as described by Stevens et al., in press.
[b]EOM (extractable organic matter) from air particles collected by filtration.
[c]Lead coefficient were from Albuquerque results.

Human Exposure Assessment Using Micromutagenesis Methods. Because the initial phases of the IACP are focusing on characterizing the principal organic mutagens and carcinogens from products of incomplete combustion, the exposure assessment component of the program is designed to characterize the important human exposure environments indoors and outdoors. Micromutagenesis assays measure exposure to mutagens from organics extracted from filtered particles and semi-volatiles collected on XAD-2 resin. The IACP samples are collected simultaneously inside and outside homes and across the airshed. Results from these studies have shown that wood stove emissions increase the concentration of mutagens indoors primarily through infiltration of outdoor air. As a result, the concentration of mutagenic activity indoors is correlated with outdoor air but is lower than the outdoor air, as shown in Table 3.

TABLE 3
Exposure to Mutagenicity in a Woodstove Impacted Neighborhood in Raleigh, NC[a]

	Relative Mutagenicity[b] from Airborne Particles		
Date	Community Site	Outside Home	Inside Home
2/27	1.0	1.0	1.0
2/26	1.7	3.1	1.0
2/13	3.6	5.1	2.9
2/14	4.7	11.2	3.0
2/20	5.7	7.5	4.6
2/21	11.1	16.3	5.1

[a]Mutagenicity measured in organic extracts of air particles in a microsuspension mutagenicity assay in *Salmonella typhimurium* as described by Lewtas et al., 1987.
[b]Mutant frequency (MF) per m^3 for each location was divided by the MF/m^3 for 2/27.

Characterization of the total organic exposures also includes measurement of volatile organic compounds, specific polycyclic aromatic hydrocarbons (Merrill, 1987; Zweidinger et al., 1987), and selected polar oxygenated and nitrated organics.

Comparative Mutagenesis Carcinogenesis and DNA Adduct Dosimetry. To develop a comparative data base for risk assessment, mutagenesis and carcinogenesis bioassays are being used to evaluate complex mixtures of ambient air and combustion source particle extracts. A comparative potency method has been developed for cancer risk assessment based on a constant relative potency hypothesis and using data from a battery of short-term mutagenesis bioassays and animal tumorigenicity studies on a series of diesel vehicle emissions (Lewtas, 1985b). The same bioassays have been used to evaluate complex emissions for which human lung cancer risk estimations are available from occupational and relatively high-dose exposures (emissions from coke ovens, roofing tar pots, and cigarette smoke), thereby providing a comparative basis for estimating lung cancer risk from emissions for which human data are not available. Recent EPA studies have expanded the combustion emission data set for which comparative mutagenesis and carcinogenesis data are available to include gasoline vehicle emissions and coal, oil, and wood combustion (Lewtas, 1985a). The IACP will further extend this data base to include comparative mutagenesis and carcinogenesis studies of composites of ambient particulate organics from the airsheds under study.

Highly sensitive methods are now being used to measure protein and DNA adducts that may result from exposures to environmental pollutants. Several combustion emission constituents including polcyclicaromatic hydrocarbons (e.g., benzo(a)pyrenes) and nitrogen-containing aromatic compounds (e.g., nitrosamines and 4-aminobiphyenyl) can be detected either as protein or DNA adducts (Harris, 1985). The postlabeling assay for DNA adducts is particularly applicable to complex mixtures, because the assay does not depend on prior identification of the specific chemical in the mixture that may form adducts (Randerath et al., 1985). DNA adducts dosimetry studies using this postlabeling assay are being conducted simultaneously in comparative microbial mutagenesis and tumorigenesis assays of airborne particle extracts from the IACP field study. These new dosimetry methods for complex mixtures may provide a critical data link between exposure and dose in human, animal, and *in vitro* studies, thereby reducing further the large uncertainties in current risk-assessment methodologies.

CONCLUSION

Although studies of complex mixtures of soots and tars in the early 1900s were the first to use animal cancer bioassays, the development of short-term genetic bioassays has fostered emerging methodologies and new assessment strategies for complex mixtures. These new strategies are characterized by their multidisciplinary nature. Methodologies developed by engineers, chemists, physicists, and biologists have been integrated into new strategies are providing evidence on the sources and nature of

airborne carcinogens. The use of bioassay methods in exposure and dosimetry assessment will provide direct data on human exposure to genotoxic airborne carcinogens. Comparative mutagenesis and animal tumorigenesis studies are being developed as an alternative risk assessment methodology for complex combustion emissions.

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